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Microarray analysis of endophyte-infected and endophyte-free tall fescue

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ABSTRACT

Many grasses have mutualistic symbioses with fungi of the family Clavicipitaceae. Tall fescue can harbor the obligate endophyte, *Neotyphodium coenophialum* that is asexually propagated and transmitted via host seeds. Total RNA was isolated from pseudostems of known endophyte-infected (E+) and endophyte-free (E−) plants and tested in triplicate on the Affymetrix Wheat Genome Array GeneChip® and Barley1 Genome Array GeneChip®. Overall 14–15% and 17–18% of the probe sets were called present on the wheat and barley chips, respectively. In order to identify genes that were specifically differentially expressed between the E+ and E− tall fescue, a combination of both barley and wheat target sequences that were differentially expressed (greater than twofold) that were similar on both chips on both barley and wheat arrays yielded 32 probe set (genes) that were differentially expressed. Tall fescue ESTs were identified for a number of the probe sets that were differentially expressed on the barley and wheat arrays. PCR primers were designed to fescue ESTs and tested to verify the expression profile observed in the microarray experiments. Some primers confirmed the expected results, although in other cases no differences were observed between the E+ and E− plants, or the results were contrary to what was expected. Our results suggest that while some differentially expressed genes were identified by this method, the cross-species hybridization appears to have significant limitations for the transcriptome analysis of tall fescue.
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Introduction

Many grasses of the subfamily Pooideae have mutualistic symbioses with fungi of the family Clavicipitaceae (Clay, 1990). Tall fescue, an important forage grass, has demonstrably greater adaptive advantages to environmental stresses when symbiotic with its fungal endophyte, *Neotyphodium coenophialum* (E+) compared to aposymbiotic tall fescue (E−) (Malinowski and Belesky, 2000; Schardl et al., 2004). The fungal symbiont benefits include physical protection, access to nutrients from the host and dissemination through the host seed (Schardl et al., 2004). The host plant benefits through the production of anti-insect, anti-nematode and anti-mammalian alkaloids, lolines, peramine and ergotoxins (Bush et al., 1997), the latter affecting livestock resulting in animal toxicosis, or staggers, resulting in significant economic losses (Hoveland, 1993). Additional benefits to the host appear to be enhanced drought tolerance, improved root growth, improved above-ground biomass accumulation, enhanced ability to acquire mineral phosphate from the soil, and improved nitrogen utilization (Malinowski and Belesky, 2000). However the molecular basis for this cross communication remains obscure, although work has implicated reactive oxygen species as one of the target signals that might be involved in the metabolic cross-talk (Tanaka et al., 2006).

Little of the molecular basis of the plant-endophyte interaction is known. A number of differentially expressed genes between E+ and E− tall fescue plants have been identified using a subtractive hybridization technique (Johnson et al., 2003). No discernable pathway or set of genes was identified using this procedure, although some potentially intriguing genes, such as omega fatty acid desaturase and a transporter gene were observed to be differentially expressed. Microarray analysis offers the opportunity to differentiate the relative expression of a large number of genes simultaneously. Although microarray platforms exist for a close relative of tall fescue, *Lolium perenne* (Ciannamea et al., 2006; Sawbridge et al., 2003), as well as an endophyte microarray (Felitti et al., 2006), their use is limited due to restricted public access. We sought to determine whether the Affymetrix microarray platform for wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) would give sufficient results to be of use with tall fescue. Cross-species hybridization using the barley chip with leaf samples of barley, wheat, oat, rice, sorghum and maize and resulted in 45%, 25%, 12%, 9%, 8% and 6%, positive calls, respectively (Close et al.,

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2004). It has been noted that there is significant DNA homology between *Lolium* species and barley and wheat (Sawbridge et al., 2003) suggesting that such an approach may be feasible.

Materials and methods

Plant material

Tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh = *Schedonorus arundinaceus* (Schreb.) Dumort] cv. KY31 endophyte-infected (E+) and endophyte-free (E−) seed were obtained from Dr. Tim Phillips (University of Kentucky) and sown in the greenhouse. The seed lot used was harvested from the field in 2004, some seed was left at room temperature to aid in the loss of the endophyte in the seed, and the remainder was stored at −20 °C to maintain viability. Plants were grown in the greenhouse under 16/8 light/dark conditions for 8 months prior to RNA isolation and were confirmed to be E+ or E− by immunoblot assays (Phytoscreen Neotyphodium Immunoblot Assay, Agrinostics, Inc., Watkinsville, GA, USA) and PCR analysis (see below). A single plant derived E+ and E− clone pair (Siegel et al., 1990) was provided by Dr. Christopher Schardl (University of Kentucky, Department of Plant Pathology, Lexington, KY, USA). This clone pair was used as it provided a genetically identical tall fescue genotype with and without the endophyte, whereas the tall fescue KY31 cultivar is a very heterogeneous and heterozygous population as tall fescue is an obligate out-crossing species. This clone pair has been previously used to characterize differential gene expression in E+ and E− tall fescue plants using a differential hybridization method (Johnson et al., 2003).

RNA isolation

Total RNA was isolated using the Trizol (Invitrogen), method from 1 to 2 cm pseudostem sections directly adjacent to the crown from five plants per replication (three replications) for both the E+ and E− plants. Multiple plants were sampled to insure that the expression profiles were representative for tall fescue with and without the endophyte. The re-suspended RNA was applied to a RNeasy® (Qiagen, Inc., Valencia, CA, USA) column and incubated with DNase (Turbo DNA-free™, Ambion Inc., Austin, TX, USA) on the column prior to elution. RNA concentrations were measured using the NanoDrop 1000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The RNA was ethanol precipitated and re-suspended to a concentration of 500 ng/μL. The integrity of RNA was checked using the Bio-Rad Experion Automated Eletrophoresis Station (Bio-Rad Laboratories, Hercules, CA, USA). Hybridization to the Affymetrix Gene chips was done at the University of Kentucky Microarray Facility (Lexington, KY, USA).

Microarray analysis

RNA was labeled and hybridized to the Affymetrix (Affymetrix, Santa Clara, CA, USA) GeneChip® Wheat Genome Array and GeneChip® Barley1 Genome Array at the University of Kentucky Microarray Facility using Affymetrix protocols for the Affymetrix GCS 3000 7G scanner. The Wheat Genome Array has 61290 probe sets including controls, and the Barley Genome Array, 22840. Analysis of the raw data was done using the GC adjusted Robust Multi-chip Analysis (GCRMA) method with the Stratagene ArrayAssist Software (Stratagene, Inc., La Jolla, CA, USA). Samples were tested in triplicate for each of the E+ and E−.

Identification of tall fescue homologues

Identification of wheat homologues from the barley probes was done by using BLASTn (Altschul et al., 1990) on the Affymetrix web site using the wheat target sequences for matches. Tall fescue homologues were identified using BLASTn on the NCBI EST database (Mian et al., 2008), and from our in house tall fescue sequences (unpublished). Tall fescue sequences with high similarity to the wheat and barley sequences (E = 1e−50 or less) were considered for further analysis. Tall fescue, barley and wheat sequences were aligned and probe locations were analyzed to verify Affymetrix probe targets.

Real-time PCR and PCR

PCR primers were constructed based on tall fescue sequences using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Rozen and Skaletsky, 2000) (Table 1). cDNA was made from the same original samples of the RNA submitted for microarray analysis, from RNA of other E+ and E− plants, and from a tall fescue E+ and E− clone pair (Siegel et al., 1990), using the Stratagene First Strand Synthesis kit (Stratagene, Inc., La Jolla, CA, USA). Target DNA was diluted to 100 ng/μL and 0.5 μL was used in a standard 25 μL PCR reaction [20 mM Tris–HCl (pH 8.0), 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 100 ng of each primer and 2 units to *Taq* DNA polymerase (Invitrogen, Life Science Technologies, Carlsbad, CA, USA)]. PCR reactions were run for 35 cycles of 55, 72, and 95 °C after an initial 5 min denaturation step at 95 °C and were terminated by 7 min at 72 °C. PCR reactions were visualized and photographed using a Kodak Gel-Logic 200 documentation system following electrophoresis on a 1% agarose gel. Control primers were: tall fescue actin (GB AY194227). To verify the presence or absence of the endophyte, the *N. coenophialum tub2* primers were utilized (Dombrowski et al., 2006). Primers were also synthesized using the sequences provided in Johnson et al. (2003) for tall fescue genes with homology to those on the Affymetrix Wheat and Barley chips.

Results

Microarray analysis

Initial results using the Affymetrix GCOS software called 14–15% and 17–18% of the probe sets as present on each the wheat and barley chips, respectively using tall fescue RNA. When the replicate data sets were combined, 5897 (9.6%) and 5623 (9.2%) probe sets were called present on the wheat chip for the E+ and E− plants, respectively. Similar results were observed for the barley array, 2127 (9.3%) and 2208 (9.7%) for E+ and E−, respectively, were called positive. This result is similar to what was reported in cross-species hybridization experiments using the barley array by Close et al. (2004). Roughly one third of the probe sets returned very low signal intensities (<100), suggesting that mRNA for these probe sets was not present in the fescue plants, or homologues for these genes are sufficiently different that no hybridization occurred. Overall, the results suggested that more genes were down-regulated in the E+ plants.

One interesting result obtained from the wheat array was that some of the probe sets between E+ and E− plants most significant for differential expression were for genes that had high similarity to fungal sequences (data not shown). These probe sets were undoubtedly constructed based on fungal contamination in the sequenced wheat EST's, however it suggests cross-hybridization to *N. coenophialum* transcripts in the E+ plants.

Table 1
Primers sequences. Primer name is synonymous with the tall fescue GenBank accession number.

Primer name	Primer sequence	GenBank or Affymetrix chip match
TF actin		
For	CGCCATCCAGGCTGTGCTTTC	Tall fescue actin (GB AY194227)
Rev	CCAGCTCCTGTTCATAGTCAAG	
Neo Btub		
For	ACTTCTTCATGGTCGGCTTC	*N. coenophialum* B-tubulin 2
Rev	CAGGTGCCTCCTCCTCATAC	
DT707657		
For	CAGCGTCGACATCAACCTC	Wheat Affymetrix: Ta.13280.1.S1_a_at
Rev	TGATCGGTTGACAGTGTCGT	
DT712844		
For	GGAGCTTGCTTGAAAGGAGA	Wheat Affymetrix: TaAffx.128545.1.S1_at
Rev	TACCAGCAGCAGCAAAAGAA	
DT689013		
For	CTCGTCCGTCGACCTGTACT	Wheat Affymetrix: TaAffx.120727.1.S1_at
Rev	CCCAGCTGCTTCAGTTTTTC	
DT685393		
For	GCGCAAATAGGGTAGCTGAG	Wheat Affymetrix: Ta.7291.1.S1_at
Rev	ACCCCATTGAGTGCTTCATC	
DT703831		
For	ACCATTTCGTCGAGCACCT	Wheat Affymetrix: TaAffx.132143.1.S1_s_at
Rev	ACGATATCCGGGTGGTAGG	
DT707383		
For	GGCAGCACTCTCACTGATCTT	Wheat Affymetrix: Ta.1991.1.S1_at
Rev	CTCCTTGAGGCCCTTCTTCT	
DT712372		
For	AATGTGGGATCTGCTCAAGG	Wheat Affymetrix: TaAffx.128712.4.S1_x_at
Rev	TCACGATCGATTCCCTTTTC	
DT715267		
For	TGCTTCATTAATAGGCTCACGA	Barley Affymetrix: HVSMEc0015H24f_at
Rev	AGGAAGGTACTGGACTTTTGGA	
DT709078		
For	CTTTCTGATCGTCTGCACCA	Barley (GB AAR29965)
Rev	AACTCACCAAAATCGCCATC	
DT681727		
For	GAGCCTCCGTTAGTCACAGC	Barley (GB AAR29964)
Rev	ACTTTGGCCAAGGAAGACCT	
DT711104		
For	AATCACCTGCGTATGCGCTTA	Barley (GB AAR29964)
Rev	CCAAGGATAGACGATGGAGTT	
DT683234		
For	TGGATATGAGGACAAGACCGA	Barley (GB AAR29967)
Rev	GAATAAAGAACAGCCCAGCA	

Comparison between the Barley and Wheat ESTs

In order to identify putative tall fescue homologues that were differentially expressed based on the microarray analysis, we chose to concentrate on a set of genes that were identified through the combination of the barley and wheat microarray experiments. The first list identified 219 probe sets that were differentially expressed on the wheat array (E+ > E− 27 and E+ < E− 192) and 76 genes that were differentially expressed on the barley microarray (E+ > E− 18 and E+ < E− 53). Thus, overall the results suggested that more genes were down-regulated by the presence of the endophyte in the E+ plants.

Since the barley array has the smaller probe set number (22840 vs 61290 for wheat), the genes identified on the barley array were used to identify homologues in the wheat array by BLASTn to the Affymetrix wheat target sequences. All but one of the barley sequences identified a positive match on the wheat array. Positive matches were then screened to identify those probe sets that also were found to be differentially expressed on the wheat microarray experiment. This resulted in a list of 32 differentially expressed probe sets with similar sequences over both the barley and wheat chips (Table 2), although in many instances the trend was similar when comparing the arrays (i.e. barley array to wheat array and wheat array to barley array), simply not declared significant or less than twofold. Once probe sets that identified the same gene, or gene family member, were combined; 11 were twofold higher in the E+ plants and 23 that were observed to be twofold lower in the E+ plants.

Analysis of fescue sequences and PCR results

In order to verify expression observed on the arrays, primers were designed to amplify a 300–600 bp fragment of the putative tall fescue homologue that had the closest match by BLASTn (E = 1e−50 or less) to a number of sequences observed to be differentially expressed on the wheat and barley arrays (Fig. 1). However, some of the PCR primers gave somewhat contradictory results. First, not all of the primers resulted in products. The reason for this result is unknown as the primers were synthesized based on the tall fescue sequences. In an attempt to determine whether those primers that

Table 2
Description of differentially expressed probe set from the Affymetrix GeneChip® wheat genome array and GeneChip® barley genome array using tall fescue RNA.

Barley Contig	Log 2	E BLASTn	Wheat probe set	Log 2	E BLASTn	Fescue EST	E BLASTx	Putative Protein ID
	E+ < E−							
Contig14115_at	−2.53	0.00E+00	Ta.3031.1.A1_at	−5.33		None	2.00E−34	Putative myb transcription factor [Oryza sativa]
Contig2265_at	−5.52	8.00E−78	Ta.3651.1.S1_at	−3.60	5.00E−162	DT712005	8.00E−78	Adhesion of calyx edges protein (ACE)
Contig2504_at	−2.07	1.00E−146	Ta.4938.1.S1_at	−1.84	9.00E−12	DT704524	7.00E−63	Putative IAA1 protein [Oryza sativa]
Contig1517_at	−1.15	1.00E−116	Ta.28528.1.S1_at	−0.89	0	DT701495	1.00E−116	Germin D [Hordeum (see also auxin-binding protein [ABP]1 L)]
Contig3391_at	−1.29	1.00E−140	Ta.9316.1.S1_at	−3.07	3.00E−163	DT706014	1.00E−140	Probable glucan 1,3-beta-glucosidase
Contig1518_at	−1.33	1.00E−174	Ta.25181.1.S1_at	−1.63	2.00E−148	DT702473	1.00E−117	Oxalate oxidase [*Triticum aestivum*]
Contig16732_at	−2.06	4.00E−78	Ta.1619.3.A1_x_at	−5.49		None	8.00E−27	Extensin-like protein [*Arabidopsis thaliana*]
Contig17136_at	−2.11	1.00E−164	Ta.3697.1.S1_at	−3.30	3.00E−120	DT689840	5.00E−40	ABC transporter family protein [*A. thaliana*]
Contig1397_at	−2.22	0.00E+00	Ta.23142.9.S1_at	−2.06	1.00E−131	DT686336	1.00E−128	Actin 1 [Avena nuda]
rbags19n19_s_at	−3.77	8.00E−26	Ta.3361.2.S1_x_at	−4.34	1.00E−12	DT693006		None
Contig11177_at	−1.07	2.00E−43	Ta.7290.1.S1_x_at	−1.61	7.00E−50	DT712184	2.00E−43	Putative myosin-like protein [Oryza sativa]
Contig4451_at	−1.39	7.00E−92	Ta.28561.1.S1_at	−1.93	1.00E−79	DT685834	1.00E−124	Cellulose synthase-3 [Zea mays]
Contig333_3_x_at	−1.79	7.00E−94	Ta.812.1.S1_x_at	−1.81	9.00E−103	DT703040	1.00E−131	Tubulin alpha-2 chain [Hordeum vulgare]
Contig5663_at	−1.64	1.00E−105	Ta.7498.2.A1_a_at	−1.75	2.00E−04	DT688051	1.00E−05	Putative fatty acid elongase [Zea mays]
Contig20165_at	−1.23	3.00E−67	Ta.10214.2.S1_at	−3.95	2.00E−152	DT709871	3.00E−67	Cellulose synthase [Populus tremuloides]
Contig600_at	−1.08	3.00E−41	Ta.28600.1.S1_a_at	−2.94		None	3.00E−92	Carboxypeptidase C [Hordeum vulgare]
Contig5363_at	−1.64	1.00E−101	Ta.6957.1.S1_at	−1.87	5.00E−133	DT715701	1.00E−112	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase alpha subunit
Contig5202_at	−1.47	3.00E−39	TaAffx.112481.1.S1_at	−1.36		None	1.00E−103	RACB protein [Hordeum vulgare]
HVSMEn0019D12r2_s_at	−1.28	1.00E−146	Ta.4878.1.S1_at	−1.27	1.00E−127	DT684167	2.00E−10	Endo-1,4-beta-glucanase Cel1 [Hordeum vulgare]
Contig9841_at	−1.40	7.00E−12	Ta.9309.3.A1_a_at	−1.50		None	7.00E−12	At1g19870 [*A. thaliana*]
Contig4896_at	−1.03	8.00E−69	Ta.8678.1.S1_a_at	−1.06	4.00E−11	DT710166	8.00E−69	Unnamed protein product [Oryza sativa]
Contig4696_at	−1.64	2.00E−67	Ta.2471.3.S1_a_at	−1.65	5.00E−116	DT711815	3.00E−93	UMP/CMP kinase a [Oryza sativa]
Contig5919_at	−1.90		Ta.7247.1.S1_at	−2.60	9.00E−12	DT688898	1.00E−124	At1g27440~unknown protein [*A. thaliana*]
	E+ > E−							
HVSMEf0001F23r2_at	1.04	1.00E−38	Ta.2009.1.S1_at	1.31	2.00E−35	DT697473	8.00E−09	Ripening-associated protein [*Musa acuminata*]
Contig4322_at	1.32	1.00E−158	Ta.2009.1.S1_at	1.31	1.00E−125	DT704489	2.00E−73	Aluminum-induced protein-like
Contig5656_at	1.27	2.00E−70	Ta.13209.1.S1_at	1.12	7.00E−10	DT704084	2.00E−70	Adenine phosphoribosyltransferase [*A. thaliana*]
Contig7508_at	0.82	1.00E−64	Ta.2396.2.S1_a_at	1.08	8.00E−111	DT711547	1.00E−64	Putative protein, At5g67370 [*A. thaliana*]
HVSMEc0015H24f_at	1.47	2.00E−99	TaAffx.80571.1.S1_at	1.46	0	DT715267	3.00E−92	NADH dehydrogenase subunit K [*Triticum aestivum*]
HVSMEc0019G06f_at	1.26	2.00E−70	TaAffx.128896.23.S1_at	1.69		None	0.012	Hypothetical protein [Picea abies]
Contig1760_s_at	1.67	1.00E−136	Ta.1983.1.S1_at	1.07	1.00E−94	DT680081	5.00E−88	g5bf [*A. thaliana*]
ChlorContig14_at	2.05		TaAffx.128896.23.S1_at	1.69	2.00E−76	DT712372	4.00E−94	Photosystem II 43 kD protein
HVSMEb0007O01f_at	1.46	1.00E−113	TaAffx.128712.4.S1_x_at	1.31	2.00E−76	DT712372	7.00E−08	Photosystem II 44 kDa (CP43) psbC precursor
ChlorContig17_s_at	1.99	2.00E−12	TaAffx.128896.23.S1_at	1.69	3.00E−103	DT715409	1.00E−129	Chloroplast 50S ribosomal protein L2
Contig378_s_at	2.54	8.00E−47	TaAFFX-Ta_18SrRNA_at	1.80	2.00E−106	DT693811	9.00E−28	rRNA intron-encoded homing endonuclease [Oryza sativa]

Wheat Probe Set	Sig	Fescue EST	PCR	Similarity
Ta.18223.1.S1_a_at	-4.898	DT705808		Transcribed locus, strongly similar to NP_916751.1 GDSL-motif lipase/hydrolase-like protein [Oryza sativa (japonica cultivar-group)
Ta.11609.2.S1_a_at	-1.054	DT702936		Transcribed locus, moderately similar to NP_565210.1 LEM3 (ligand-effect modulator 3) family protein / CDC50 family protein [Arabidopsis thaliana]
TaAffx.120727.1.S1_at	1.1495	DT712844		Transcribed locus, moderately similar to XP_470974.1 [Oryza sativa (japonica cultivar-group)]
TaAffx.30463.1.S1_at	1.153	DT689013		Similar to DUF1295 proteins
TaAffx.131520.1.S1_s_at	1.2732	DT685393		Transcribed locus, weakly similar to NP_914269.1 [Oryza sativa (japonica cultivar-group)]
Ta.28243.1.A1_x_at	1.4874	DT703831	No Product	Transcribed locus, moderately similar to NP_921943.1 putative cyanase [Oryza sativa (japonica cultivar-group)]
Ta.1991.1.S1_at	1.608	DT707383		Dehydrin COR410
TaAffx.128712.2.S1_s_at	1.6585	DT712372		Photosystem II 44 kDa reaction center protein
TaAffx.80571.1.S1_s_at	1.6739	DT715267		NAD(P)H-quinone oxidoreductase chain K, chloroplast (NAD(P)H [Triticum aestivum]
Neo Tubulin	ND	None		Neotyphodium tubulin primers
Actin	ND	AY194227		Tall fescue actin

Fig. 1. RT-PCR analysis of putative differentially expressed tall fescue genes based on microarray. E+ and E− indicate combined RNA from a number of endophyte-infected (E+) and endophyte-uninfected (E−) plants. Tall fescue clone pairs, 278 and 279, are lines derived from a single genotype that are endophyte-infected (278) and endophyte-uninfected (279). Primers to the *N. coenophialum* β-*tubulin* 2 were used to verify presence or absence of the endophyte, and the tall fescue actin primers were used as the control.

did not produce bands were due to problems with the sequences or the primers, new primers were synthesized for five genes based on the wheat probe sequences that matched the fescue sequences. In none of the cases was a PCR band observed. Since the primers were synthesized based on tall fescue EST sequences, it is expected that these are expressed genes, but this result suggests that these particular genes were not expressed in the tissues sampled.

Second, of the primers that produced bands, only half of those gave results similar to those observed in the microarray analysis. In most of these cases no differences were observed in the PCR band intensities between the E+ and E− plants (as seen for DT705808 and DT685393 in Fig. 1). In some instances the PCR results were reversed from what was observed from the microarray experiments. This was the case for the primers used to detect the cellulose synthase-like genes (Fig. 2). As expression of some of the CES genes tend to be similarly regulated (Burton et al., 2004), we tested additional tall fescue CesA-like EST homologues identified in the NCBI database. Of those that gave positive PCR results, all were observed to have higher expression in the E+ plants (Fig. 2). These results suggest that the mRNA detected in the microarray by these probes was probably not a CES-like gene. To further elucidate the possible reason for this contradictory result, tall fescue ESTs were used to map on the wheat and barley probe sets. None of the probe sets matched the tall fescue sequences since the probe sets were based primarily in the 3′ UTR regions, and the Blastn match was based on the coding sequences further upstream (not shown). Thus it is probable that while these probe sets serve to identify the wheat and barley genes CES genes, it is unknown which genes are being identified in tall fescue.

To further verify that expression analysis was a comparison between the E+ and E− plants, RNA was isolated from a tall fescue clone pair previously used to analyze differential expression by

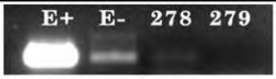
Fescue EST	PCR	Description
DT709078		Moderately similar to NP_567564.1 cellulose synthase, catalytic subunit (IRX1) Hv AAR29965-like
DT681727		cellulose synthase, catalytic subunit Hv AAR29964-like
DT711104		cellulose synthase, catalytic subunit Hv AAR29964-like
DT683234		cellulose synthase, catalytic subunit Hv AAR29967-like

Fig. 2. RT-PCR analysis of cellulose synthase-like genes from tall fescue. Fescue EST sequences were obtained from GenBank. E+ and E− indicate combined RNA from a number of endophyte-infected (E+) and endophyte-uninfected (E−) plants. Tall fescue clone pairs, 278 and 279, are lines derived from a single genotype that are endophyte-infected (278) and endophyte-uninfected (279).

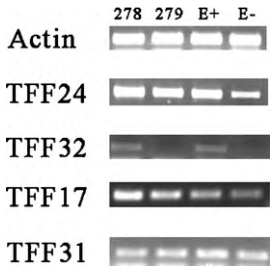


Fig. 3. RT-PCR analysis using primers derived from Johnson et al. (2003). E+ and E− indicate combined RNA from a number of endophyte-infected (E+) and endophyte-uninfected (E−) plants. Tall fescue clone pairs, 278 and 279, are lines derived from a single genotype that is endophyte-infected (278) and endophyte-uninfected (279).

subtractive hybridization (Johnson et al., 2003). The 278 (E+) and 279 (E−) clone pair provides a genetically identical background for expression analysis where the only difference should be the presence or absence of the endophyte (Siegel et al., 1990). PCR analysis was done using the same primers used above, and in most instances the results observed were similar to those from wild-type E+ and E− plants (Figs. 1 and 2). Twenty-seven plant sequences published by Johnson et al. (2003) were screened against the Affymetrix barley and wheat target sequences using BLASTn. Six matches were found in the barley microarray set, and five on the wheat, two in common (E = 1e−50 or less). None of the probe sets on either the barley or wheat arrays were found to give a significantly different signal between the E+ and E− plants (data not shown). PCR verification using the published primers yielded similar results as Johnson et al. (2003) in four of the five primer combinations tested, confirming the previously observed PCR results (Fig. 3).

Discussion

Here we report on the use of the Affymetrix Array GeneChips from barley and wheat to assess general expression of E+ and E− tall fescue. Overall, using the non-homologous chips resulted in poor detection levels, although these are in line with the results that would be expected from cross-species hybridization experiments on these chips (Close et al., 2004). Regardless, the microarray experiments allowed for the analysis of several thousand putative tall fescue cDNAs. Significant differences were found between the E+ and E− plants in both the barley and wheat microarray experiments. The use of the combined barley and wheat microarray chip confirmed that similar genes were detected using the different platforms and probably gave the best subset of differentially expressed genes that were present in the E+ and E− plants analyzed. Presently we are uncertain as to the precise tall fescue gene products that were found to be differently expressed on the arrays since the closest Blastn matches could not always be confirmed by PCR using tall fescue specific primers. While Close et al. (2004) have reported values comparable to what was observed in our experiment in cross-species hybridization experiments, follow up validation experiments were not done. Cross-species hybridization experiments have been shown to be successful for gene identification using closely related species, *Arabidopsis halleri* on *Arabidopsis thaliana* arrays (Becher et al., 2004) and *Medicago sativa* on *Medicago truncatula* arrays (Tesfaye et al., 2006). Our results demonstrate the necessity for validation when more distantly related species are tested.

There did not appear to be any specific group of related proteins that were coordinately up- or down-regulated by the presence of the endophyte. This is probably more a feature that a low number of positive genes (probe sets) were found to be differentially expressed. The only group of similar type of proteins that appeared to be similarly regulated were chloroplast derived genes (CP43 and ndhK). Another subunit of the NAD(P)H dehydrogenase complex, ndhE gene, was also observed to be expressed greater than twofold ($\log 2 = 1.04$), but was not considered significant ($P = 0.057$ on the wheat array; data not shown). The two-dehydrogenase genes are derived from different plastid polycistronic messages that include genes for other components of the dehydrogenase complex (one containing ndhH, ndhS, ndhI, ndhG, ndhE and ndhD, and ndhC, ndhK and ndhJ on the other). The apparent increase in hybridization to the plastid derived gene probe sets for the E+ plants is interesting in the fact that associations of the endophyte has been correlated with decreased net photosynthesis in tall fescue (Belesky et al., 1987) as well as ryegrass (Spiering et al., 2006). Thus this apparent increase might be an indication that these polyadenylated plastid transcripts are targeted for degradation in the E+ plants (Schuster et al., 1999). And while the exact role of the chloroplast dehydrogenase complex is not understood, it has been observed to increase in response to photo-oxidative stress, and shares similarities to pathogen infection response (Casano et al., 2001). With the observations that reactive oxygen species (ROS) may be involved in the plant-endophyte cross-talk in the endophyte (Tanaka et al., 2006), the NAD(P)H complex may play a role in the plant. Thus while the endophyte has not been associated with any long term detrimental effect on photosynthesis and growth, these results may suggest some mechanism for decrease in expression of some plastid genes in response to the presence of the endophyte.

It was interesting to note that a larger number of putative tall fescue genes appeared to be down-regulated than up-regulated in the E+ plants in the microarray analysis. And while this may be a general feature of the presence of the endophyte, it is more likely due to the lack of tall fescue specific genes represented on the array. It has been reported that approximately 20–30% of ryegrass and tall fescue sequences do not appear to have matching sequences from other species in the databases (Sawbridge et al., 2003; Zhang et al., 2005), and these would not be included in the barley or wheat array. However, we also observed that a number of genes that were supposedly down-regulated in the E+ plants could not be confirmed by PCR, either due to lack of PCR product or that the PCR results did not support the microarray data. It is presently unknown which fescue cDNAs these probes identified on the microarray as many the probe sets on the chips are based on the 3′ ends of the barley and wheat cDNAs, where there is the largest divergence between species.

One serendipitous reverse positive PCR reaction identified the cellulose synthase (CES) genes. The region on array probe set did not match any tall fescue sequences, but the upstream part of the putative consensus sequence. However, when we followed up with additional analysis of other CES-like genes, it was noted that all yielded higher expression in the E+ plants. The rationale of the observed differential expression of these genes is presently unknown and merits further study.

In conclusion, we have tested the Wheat Genome Array and GeneChip® Barley Genome Array with E+ and E− tall fescue RNA for expression. Our results demonstrated that the presence of the endophyte affected tall fescue gene expression in the pseudostem tissues indicating that the plant does “respond” to the presence of the endophyte. Unfortunately the non-specificity of the hybridization using the Affymetrix Wheat Genome and GeneChip® Barley Genome arrays precluded a comprehensive transcriptome analysis, and will have to await the availability of genomic tools more specific to tall fescue.

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